Br. J. Pharmacol. (1994), **113**, 1008–1014

Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone

[1]Jane A. Mitchell, *Maria G. Belvisi, †Pravit Akarasereenont, *Richard A. Robbins, *O.-Jung Kwon, †Jamie Croxtall, J., *Peter J. Barnes & †John R. Vane

Departments of Applied Pharmacology and *Thoracic Medicine, The National Heart and Lung Institute, Dovehouse Street, London SW3 6LD and †The William Harvey Research Institute, Saint Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

1 Cyclo-oxygenase metabolizes arachidonic acid to prostaglandin H_2 (PGH_2) and exists in at least two isoforms. Cyclo-oxygenase-1 (COX-1) is expressed constitutively whereas COX-2 is induced by lipopolysaccharide (LPS) and some cytokines *in vitro* and at the site of inflammation *in vivo*. Epithelial cells may be an important source of prostaglandins in the airways and we have, therefore, investigated the expression of COX-1 or COX-2 isoforms in primary cultures of human airway epithelial cells or in a human pulmonary epithelial cell line (A549).

2 COX-1 or COX-2 protein was measured by western blot analysis using specific antibodies to COX-2 and selective antibodies to COX-1. The activity of COX was assessed by the conversion of either endogenous or exogenous arachidonic acid to four metabolites, PGE_2, $PGF_{2\alpha}$, thromboxane B_2 or 6-oxo $PGF_{1\alpha}$ measured by radioimmunoassay. Thus, COX-1 or COX-2 activity was measured under two conditions; initially the accumulation of the COX metabolites formed from endogenous arachidonic acid was measured after 24 h. In other experiments designed to measure COX activity directly, cells were treated with cytokines for 12 h before fresh culture medium was added containing exogenous arachidonic acid (30 μM) for 15 min after which COX metabolites were measured.

3 Untreated primary cells or A549 cells contained low amounts of COX-1 or COX-2 protein. Bacterial LPS (1 $\mu g\ ml^{-1}$ for 24 h) induced COX-2 protein in the primary cells, a process which was enhanced by interferon-γ, with no further increase in the presence of a mixture of cytokines (interleukin-1β, tumour necrosis factor-α and interferon-γ, 10 $ng\ ml^{-1}$ for all). In contrast, A549 cells contained only low levels of COX-2 protein after exposure to LPS or LPS plus interferon-γ, but contained large amounts of COX-2 protein after exposure to the mixture of cytokines.

4 Untreated human pulmonary primary cells or A549 cells released low levels of all COX metabolites measured over a 24 h incubation period. This release was enhanced by treatment of either cell type with the mixture of cytokines (interleukin-1β, tumour necrosis factor-α and interferon-γ, 10 $ng\ ml^{-1}$ for all). PGE_2 was the principal COX metabolite released by cytokine-activated epithelial cells. The release of PGE_2 induced by cytokines occurred after a lag period of more than 6 h.

5 The glucocorticosteroid, dexamethasone (1 μM; 30 min prior to cytokines) completely suppressed the cytokine-induced expression of COX-2 protein and activity in both primary cells and A549 cells.

6 In experiments where COX-2 activity was supported by endogenous stores of arachidonic acid, treatment of A549 cells with interleukin-1β but not tumour necrosis factor-α or interferon-γ alone caused a similar release of PGE_2 to that seen when the cytokines were given in combination. However, both interleukin-1β and necrosis factor-α alone produced similar increases in COX-2 activity (measured in the presence of exogenous arachidonic acid) as seen when the mixture of interleukin-1β, tumour necrosis factor-α and interferon-γ were used to stimulate the cells.

7 These findings show that COX-2 expression correlates with the exaggerated release of prostaglandins from cytokine-activated human pulmonary epithelial cells and that the induction of the enzyme is suppressed by a glucocorticosteroid. These findings may be relevant to inflammatory diseases of the lung, such as asthma.

Keywords: Prostaglandins; inflammation; cytokines; endotoxin; nitric oxide; asthma; steroid; cyclo-oxygenase; cytokines; pulmonary epithelium

Introduction

Cyclo-oxygenase (COX) converts arachidonic acid to prostaglandin H_2 (PGH_2) (Hamberg *et al.*, 1974) which is then further metabolised to various prostaglandins, prostacyclin and thromboxane A_2. COX exists in at least two isoforms (Xie *et al.*, 1991). COX-1 is expressed constitutively in endothelial cells (Mitchell *et al.*, 1993a) and is probably responsible for the production of prostaglandins under physiological conditions (Vane, 1994). COX-2 is induced by pro-inflammatory stimuli, including mitogens (O'Banion *et al.*, 1992), cytokines (Maier *et al.*, 1990) and bacterial lipopolysaccharide (LPS; Lee *et al.*, 1992; Mitchell *et al.*, 1993a) in cells *in vitro* and in inflamed sites *in vivo* (Vane *et al.*, 1994). COX-1 shares approximately 60% homology with COX-2 (Xie *et al.*, 1991; Hla & Neilson, 1992). COX-1 and COX-2 are inhibited by nonsteroid anti-inflammatory drugs such as aspirin and indomethacin, with some selectivity towards COX-1 (Meade *et al.*, 1993; Mitchell *et al.*, 1993a).

The role of the lung in prostaglandin metabolism has been extensively studied. Indeed, COX preparations from the lung were used to demonstrate the mechanism of action of non-steroid anti-inflammatory drugs (Vane, 1971). Perfused lungs from different species release large amounts of prostaglandins

[1] Author for correspondence at Department of Applied Pharmacology.

from both the vasculature and airways in response to agonists, such as bradykinin, angiotensin and endothelins (Piper & Vane, 1969; Dawson *et al.*, 1976; Bakhle *et al.*, 1985; de Nucci *et al.*, 1988). Pulmonary epithelial cells are at the interface between the environment and the delicate structures of the lung. The airway epithelium acts as a physical barrier but is increasingly recognised as a source of lipid and peptide mediators that may modulate pulmonary inflammation. Indeed epithelial cells from the airways produce PGE_2 and $PGF_{2\alpha}$ in response to bradykinin and the calcium ionophore, A23187 (Churchill *et al.*, 1989).

The regulation of the different isoforms of COX in pulmonary tissue is not known, although human pulmonary macrophages express COX-2 after stimulation with LPS (Hempel *et al.*, 1994). This may be of particular importance in the understanding of pulmonary inflammatory conditions such as asthma. Cytokines influence pulmonary epithelial cell function via an action on specific receptors (see Barnes, 1994) and airway epithelial cells express a number of inflammatory cytokines (Devalia & Davies, 1993; see Barnes, 1994), some of which are known to cause the induction of COX-2 in other cell types (Thiemerman *et al.*, 1993). We have, therefore, used human pulmonary epithelial cells exposed to different pro-inflammatory cytokines to study changes in COX isoforms. We have used a mixture of cytokines previously demonstrated to induce nitric oxide synthase in airway cells (Robbins *et al.*, 1994). In addition we have assessed the effects of the anti-inflammatory steroid, dexamethasone on changes in both the release of COX metabolites and the expression of COX-2 protein. Some of these results have been published in abstract form (Mitchell *et al.*, 1994).

Methods

Tissue culture

Isolation of human airway epithelial cells Trachea or main bronchi were obtained from either heart or heart and lung transplantation donors. These tissues were immersed in cold Hank's balanced salt solution (HBSS) and immediately transported on ice. The cartilaginous parts of trachea or main bronchi were excised, trimmed, washed and incubated overnight at 4°C in 0.1% protease (type 14) prepared in calcium/magnesium free HBSS.

Tracheal-bronchial epithelial cells were isolated by flushing the luminal side with HBSS containing 10% heat inactivated foetal calf serum (FCS). The cells were centrifuged at 270 *g* for 10 min, resuspended in HBSS containing 10% FCS, filtered through sterile 100 μm mesh, and centrifuged again the resulting pellet was resuspended in serum-free, hormone supplemented Ham's F12 medium (complete F12) containing penicillin (100 u ml^{-1}) streptomycin (100 μg ml^{-1}), amphotericin B (2 μg ml^{-1}), L-glutamine (2 mM), insulin (5 μg ml^{-1}), transferrin (5 μg ml^{-1}), epidermal growth factor (25 ng ml^{-1}), endothelial cell growth supplement (15 μg ml^{-1}), hydrocortisone (1 μM), cholera toxin (10 ng ml^{-1}) and retinoic acid (0.1 μM). The cell suspension was again centrifuged and resuspended in complete F12 medium. Cell counts were performed with a Neuubauer haemocytometer; cell viability was assessed by trypan blue dye exclusion and was greater than 95%.

Primary culture of human tracheo-bronchial airway epithelial (HTBE) cells Dissociated HTBE cells were cultured as previously described (Churchill *et al.*, 1989; Kwon *et al.*, 1994a). Cells were plated at a density of $1-3 \times 10^4$ cm^{-2} onto the culture plates (6-well) coated with collagen gel (Vitrogen 100). The culture medium was changed after 24 h and every 2–3 days thereafter. Cultured epithelial cells were nearly confluent after 10 days of culture and showed the typical cobblestone appearance of bronchial epithelial cells (Churchill *et al.*, 1989). When the cells were nearly confluent fresh F12 medium (Ham's F12) containing only L-glutamine and antibiotic was added together with test compounds.

Culture of A549 cells The human pulmonary epithelial cancer cell line (A549), derived from lung alveolar carcinoma and representative of epithelial cells, was purchased from American Type Culture Collection (Rockville, MD). A549 cells spontaneously produce PGE_2 which acts as an autocrine growth regulator (Croxtall & Flower, 1992). Furthermore, this release is enhanced by exposure to growth factors (Croxtall *et al.*, 1993). Glucocorticoid treatment results in a complete suppression of PGE_2 release (Croxtall & Flower, 1992). In previous studies comparing A549 with primary HTBE cells, both cell types were shown to be epithelial in origin (Kwon *et al.*, 1994b). The A549 cell line, therefore, is a convenient model for the investigation of COX expression. A549 cells were grown on 35 mm 6-well culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, L-glutamine (2 mM), penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). When the A549 cells were confluent, they were washed and incubated in DMEM without FCS together with test compounds.

Measurement of COX activity

COX metabolites were measured by radioimmunoassay (Salmon, 1978) for 6-oxo $PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and thromboxane B_2. Antibodies to PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 had less than 5% cross reactivity with all other prostaglandins measured. Antibody to 6-oxo-$PGF_{1\alpha}$ had less than 5% cross reactivity to prostaglandin $F_{2\alpha}$ and thromboxane B_2 and 17% cross reactivity to PGE_2. For experiments designed to measure the release of COX metabolites from endogenous arachidonic acid, cells were treated with either LPS (1 μg ml^{-1}), tumour necrosis factor-α (TNFα; 10 ng ml^{-1}), interleukin 1β (IL-1β; 10 ng ml^{-1}), interferon-γ (IFNγ; 10 ng ml^{-1}) or LPS together with IFNγ (1 μg ml^{-1} and 10 ng ml^{-1}) or a mixture of IL-1β, TNFα and IFNγ (10 ng ml^{-1} for all) for 3, 6, 12 or 24 h and the medium removed for radioimmunoassay. In separate experiments designed to measure COX 'activity', cells were treated with different cytokines for 12 h after which time fresh medium was added containing arachidonic acid (30 μM) for 15 min at 37°C and then removed for radioimmunoassay. In some experiments, dexamethasone (1 μM) was added 30 min before the addition of LPS, IL-1β, TNFα or IFNγ for 24 h and the predominant COX metabolite (PGE_2) measured.

Western blot analysis

In separate experiments HTBE cells or A549 cells were grown in 6-well plates as above. After exposure to test compounds the medium culture was removed and the cells incubated with extraction buffer (Tris, 50 mM; EDTA, 10 mM; Triton X-100, 1% v/v; phenylmethylsulphonyl fluoride, 1 mM; pepstatin A, 0.05 mM and leupeptin, 0.2 mM) with gentle shaking. The cell extract was then boiled (10 min) in a ratio of 1:1 with gel loading buffer (Tris, 50 mM; SDS, 10% w/v; glycerol, 10% v/v; 2-mercaptoethanol 10% v/v and bromphenol blue, 2 mg ml^{-1}). Samples were centrifuged at 10,000 *g* for 2 min before being loaded onto gradient gels (4–12% Tris-glycine; Novex) and subjected to electrophoresis (1.5 h at 125 V). The separated proteins were transferred to nitrocellulose (BIORAD; 1 h at 200 V). After transfer to nitrocellulose, the blot was incubated in blocking solution (dried minimal-fat milk 25 g and Tween-20 1.25 ml in PBS solutions 500 ml) for 1 h and then primed with rabbit antibody raised to murine COX-2 (Cayman Chemical Company, MI, U.S.A.) for 1 h. There was no detectable cross reactivity of the COX-2 antibody with COX-1 (Mitchell *et al.*, 1993a). In some experiments blots were primed with rabbit antibody raised to ovine COX-1 (a generous gift from

K. Wu, Houston, TX, U.S.A.) for 1 h. The COX-1 antibody has approximately 10% cross reactivity with COX-2 (Mitchell *et al.*, 1993a). The blot was then incubated with an anti-rabbit IgG developed in sheep, linked to alkaline phosphatase conjugate for 1 h. Finally, the blot was developed for approximately 5 min with premixed solution containing 5-bromo-4-chloro-3-indeolyl phosphate (0.56 mM), nitroblue tetrazolium, (0.48 mM), Tris (10 mM) and $MgCl_2$ (59.3 mM) at pH 9.2. The detection limit of protein in cell extract was 1–10 ng. The band, which was visualised at approximately 70 kDa was quantified using a laser densitometer with Quantity-One software (PDI, New York, NY, U.S.A.). Individual band density values for each point were expressed in arbitrary OD units.

Statistical analysis

Results are shown as mean ± s.e.mean from n determinations (wells) from at least three separate plates. Unpaired two tailed tests or one way analysis of variance were used as appropriate to determine the significant differences between means and P values of less than 0.05 were taken as statistically significant.

Materials

Recombinant human TNFα was purchased from British Biotechnology (Oxford, UK), Vitrogen 100 from Collagen Co, Ham's F12 medium, DMEM, pre-mixed penicillin-streptomycin and L-glutamine from ICN Flow (High Wycome, UK); FCS from Sera Lab (Crawley Down, UK). All other materials were purchased from Sigma Chemical Company (Poole, Dorset, UK), unless otherwise stated.

Results

Characterization of the isoforms of COX present in human pulmonary epithelial cells

Untreated HTBE or A549 cells contained low or undetectable levels of COX-1 ($n = 3-6$; data not shown) or COX-2 (Figure 1 and 2 respectively). However, after exposure of either cell type to a mixture of cytokines, IL-1β, TNFα plus IFNγ (all at 10 ng ml^{-1}) for 24 h there was an induction of COX-2 protein (Figure 1 and Figure 2; $n = 3-6$). The levels of COX-2 protein induced in A549 cells was greater than that induced in the HTBE cells. Treatment of either cell type with LPS (1 μg ml^{-1}) alone did not induce the expression of COX-2 protein. However, HTBE cells, but not A549 cells, contained detectable levels of COX-2 protein after treatment with LPS together with IFNγ (Figure 1). Pretreatment of A549 cells with dexamethasone (1 μM; 30 min prior to the cytokine mix) almost abolished the induction of COX-2 (Figure 3).

Release of COX metabolites by HTBE or A549 cells from endogenous stores of arachidonic acid

Untreated HTBE or A549 cells released low levels of all COX metabolites measured (6-oxo $PGF_{1\alpha}$, PGE_2, thromboxane B_2 or $PGF_{2\alpha}$ <0.1 ng ml^{-1} for A549 or primary cells, $n = 15$; Figure 4). Incubation of A549 cells or HTBE cells with either LPS (1 μg ml^{-1}) alone or together with IFNγ (10 ng ml^{-1}) did not cause detectable release of COX metabolites (data not shown; $n = 3$). However, incubation of either cell type for 24 h with a mixture of IL-1β, TNFα and IFNγ (10 ng ml^{-1} for all) caused the accumulation of PGE_2 (10.09 ± 0.63 ng ml^{-1} for A549 cells and 0.23 ± 0.04 ng ml^{-1} for HTBE cells, $n = 6-15$) $PGF_{2\alpha}$ (4.03 ± 0.16 ng ml^{-1} for A549 cells and undetectable levels for HTBE cells), 6-oxo $PGF_{1\alpha}$ (3.07 ± 0.12 ng ml^{-1} for A549 cells and undetectable HTBE cells). Thromboxane B_2 was not formed in detectable amounts by either cell type (Figure 4). The treatment of A549 cells with a combination of IL-1β, TFNα and IFNγ (10 ng ml^{-1} for all) for 3, 6, and 24 h resulted in a time-dependent release of PGE_2, which was first significant at 6 h after the addition of the cytokines (Figure 5; $n = 3-15$).

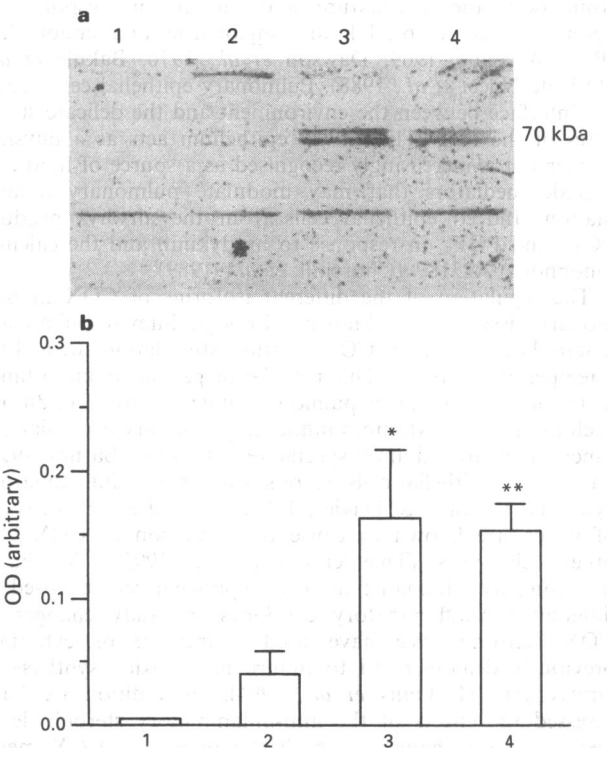

Figure 1 The expression of COX-2 protein in HTBE cells after treatment with cytokines for 24 h. (A) Shows a Western blot using a specific antibody to COX-2 which recognised a doublet of approximately 70 kDa. Each lane was loaded with 1 μg of protein. Lane 1 shows extracts from untreated cells, lane 2 cells treated with LPS (1 μg ml^{-1}) lane 3 treated with LPS and IFN-γ (10 ng ml^{-1}), lane 4 treated with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1}; lane 4); (b) shows the means ± s.e.mean of the optical density (OD) of COX-2 bands from 3 separate experiments. P values are calculated between treated and untreated groups by unpaired two-tailed t test (*$P<0.05$; **$P<0.01$). In separate experiments, no COX-1 was detected in cell extracts from any of the above treatments ($n = 3$; data not shown). For abbreviations, see text.

Characterization of the regulation by cytokines of the release of COX metabolites from endogenous arachidonic acid

A549 cells treated with a mixture of cytokines released larger amounts of COX metabolites and expressed greater amounts of COX-2 protein than HTBE cells, A549 cells were used, therefore, to characterize further the regulation of COX-2 in human pulmonary epithelial cells by specific cytokines. A549 cells treated with IL-1β (10 ng ml^{-1}) for 24 h showed a more than 30 fold increase in the release of the major COX metabolite, PGE_2 (from 0.27 ± 0.05 ng ml^{-1} to 10.84 ± 0.43 ng ml^{-1}; $n = 3$; Figure 6). However, TNFα (10 ng ml^{-1}) or IFNγ (10 ng ml^{-1}) had no significant effect on the release of PGE_2 by A549 cells ($n = 3$). The release of PGE_2 in response to IL-1β or the cytokine mixture was significantly reduced by pretreatment of the cells with dexamethasone (to 0.3 ± 0.03 ng ml^{-1} or 0.15 ± 0.01 ng ml^{-1} respectively; $n = 3-6$; Figure 6).

In A549 cells COX activity (measured in the presence of exogenous arachidonic acid; 30 μM; 15 min) was elevated at

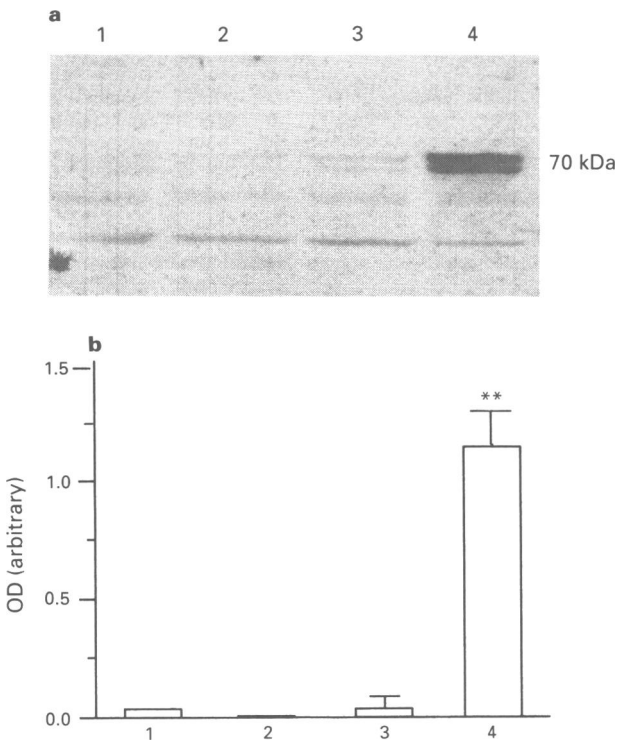

Figure 2 The expression of COX-2 protein in A549 cells after treatment with cytokines for 24 h. (A) Shows a Western blot using a specific antibody to COX-2 which recognised a doublet of approximately 70 kDa. Each lane was loaded with 1 μg of protein. Lane 1 shows extracts from untreated cells, lane 2 cells treated with LPS (1 μg ml^{-1}), lane 3 treated with LPS and IFN-γ (10 ng ml^{-1}), lane 4 treated with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1}; lane 4); (b) shows the means ± s.e.mean of the optical density (OD) of COX-2 bands from 3 separate experiments. *P* values are calculated between treated and untreated groups using unpaired two-tailed *t* test (**$P<0.01$). In separate experiments no COX-1 was detected in cell extracts from any of the above treatments ($n=3$; data not shown). For abbreviations, see text.

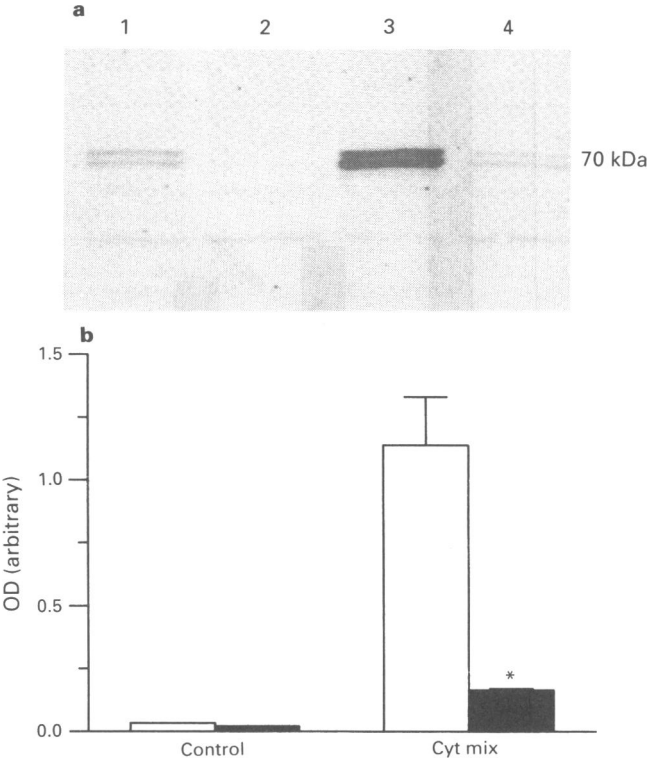

Figure 3 The effect of dexamethasone on the induction of COX-2 in A549 cells. (a) Shows a typical Western blot using a primary antibody to COX-2. Each lane was loaded with 1 μg of protein. Lane 1 contains extract from untreated cell, lane 2 from cells treated with dexamethasone (1 μM; for 24 h), lane 3 cells treated with cytokine mixture (Cyt mix; IL-1β, TNFα and IFNγ all at 10 ng ml^{-1} for all, for 24 h) and lane 4 cells treated with dexamethasone 30 min prior to treatment with the cytokine mixture; (b) shows the means ± s.e.mean of the OD of COX-2 bands from 3 separate determinations, where the open columns represent cells treated with saline and the solid columns cells treated with dexamethasone. *P* values are calculated between dexamethasone and vehicle-treated cells (**$P<0.01$ by unpaired two-tailed *t* test). For abbreviations, see text.

12 h after the addition of either a combination of IL1-β, TNFα and IFN-γ, or IL1-β alone ($n=3$; Figure 7). In contrast to the lack of effect of TNFα on the release of PGE_2 from endogenous stores of arachidonic acid, TNFα caused a significant induction of COX-2 activity as measured in the presence of exogenous arachidonic acid (Figure 7).

Discussion

Pro-inflammatory cytokines, such as TNFα or IL-1β regulate the release of several inflammatory mediators by inducing the expression of synthesizing enzymes. Thus, a mixture of IL-1β, TNFα and IFNγ induces pulmonary epithelial cells to express nitric oxide synthase (Robbins *et al.*, 1994) and LPS causes the co-induction of nitric oxide synthase and COX-2 in murine macrophages (Mitchell *et al.*, 1993b). We have extended these studies and demonstrated that pro-inflammatory cytokines stimulate human pulmonary epithelial cells to release COX metabolites, to express increased COX activity and to express COX-2 protein. No detectable COX-1 was present in either untreated or stimulated cells. In previous studies, a low release of PGE_2 was detected in unstimulated cells (Croxtall & Flower, 1992). However, these authors used a lower ratio of culture medium to cells leading to a greater concentration of metabolites. We found that cytokine-activated HTBE released lower levels of COX metabolites and contained less COX-2 protein than the human pulmonary cell line, A549. In addition, HTBE cells but not A549 cells expressed COX-2 protein after exposure to LPS and IFNγ. In primary cell cultures it was necessary to include the steroid hydrocortisone, which discourages the growth of contaminating fibroblasts. Dexamethasone abolishes the induction of COX-2 and the necessary inclusion of hydrocortisone may, therefore, explain the relative low level of induction in primary cells. In addition, the growth of HTBE cells, but not A549 cells, is suppressed by serum (Barnes & Robbins, unpublished observations). For this reason HTBE cells were serum-deprived at the time of the addition of cytokines. A lack of serum greatly reduces the ability of pulmonary epithelial cells to release prostaglandins (Churchill *et al.*, 1989) so this could be an additional reason for the differences seen between HTBE cells and A549 cells. However, our studies clearly demonstrate that human airway epithelium expresses COX-2 in response to pro-inflammatory cytokines. Others have demonstrated that extracts of human lung contain messenger RNA for COX-2, although the cellular origin was not identified (Neil & Ford-Hutchinson, 1993).

Human pulmonary epithelial cells released predominantly PGE_2 when induced to express COX-2. This finding is in agreement with previous studies, showing that PGE_2 is a major metabolite released from human epithelial cells stimulated with bradykinin or calcium ionophore (Churchill *et al.*, 1989). The induction of COX-2 in other cell types, such as endothelial cells, macrophages (Mitchell *et al.*, 1993a,b; Akarasereenont *et al.*, 1994) and fibroblasts (Kujubu *et al.*,

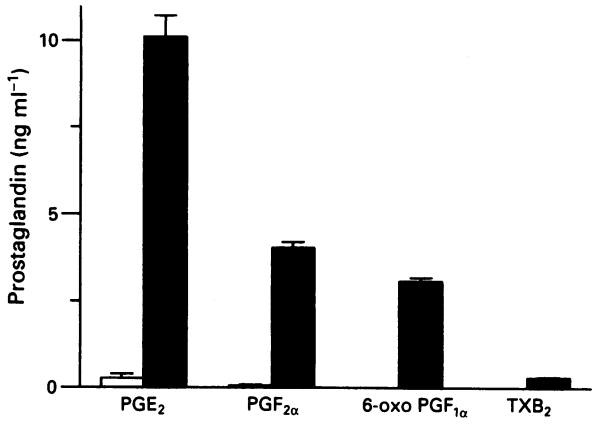

Figure 4 Release of different COX metabolites by HTBE (open columns) or A459 cells (solid columns) after treatment with a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1} for 24 h). Untreated cells released undetectable amounts of COX metabolites. The data represent the mean ± s.e.mean for 3–9 determinations. For abbreviations, see text.

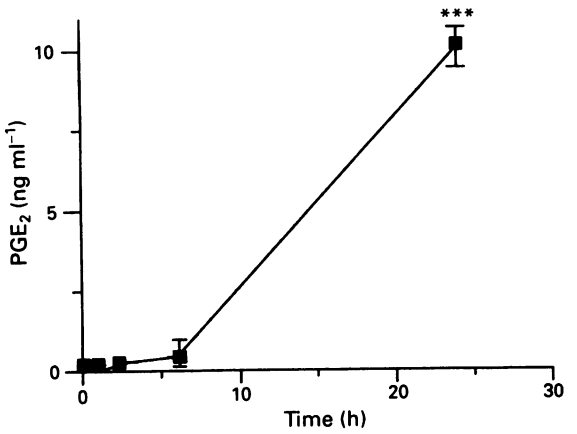

Figure 5 Time-dependent release of PGE_2 from A549 cells after the addition of a mixture of cytokines (IL-1β, TNFα, IFNγ all at 10 ng ml^{-1}). Culture medium was removed at 1, 3, 6 and 24 h after the addition of cytokines (■). No PGE_2 was detected in culture medium removed from cells 24 h after the addition of vehicle. The data represent the mean ± s.e.mean for 3–9 determinations. A significant increase (***$P<0.001$) in the amount of PGE_2 released by cells treated with cytokines compared to cells treated with vehicle (for 24 h) was calculated by one way analysis of variance. For abbreviations, see text.

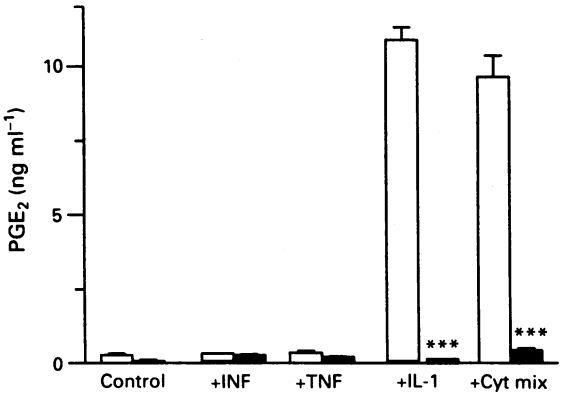

Figure 6 The figure shows the release of PGE_2 by A549 cells treated with either vehicle (Control), IL-1β, TNFα, IFNγ or a mixture of cytokines (Cytmix, IL-1β, TNFα, IFNγ all at 10 ng ml^{-1}) for 24 h (open columns). The solid columns shows the release of PGE_2 by A549 cells treated with dexamethasone (1 μM) 30 min prior to the addition of cytokines. The data represent the mean ± s.e.mean for 3–9 determinations. Significant differences in the release of PGE_2 by cells treated with dexamethasone or vehicle were calculated by an unpaired two-tailed t test (***$P<0.001$). For abbreviations, see text.

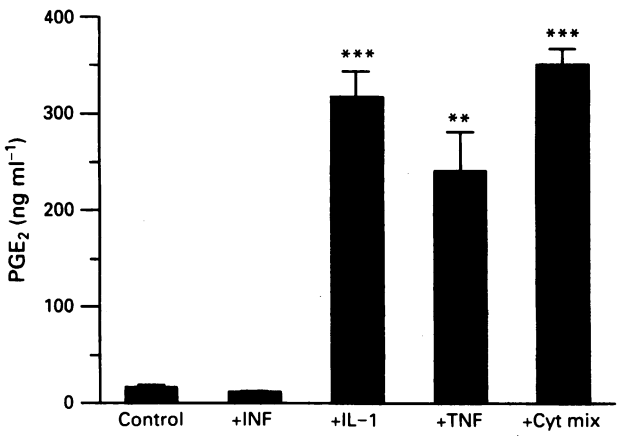

Figure 7 Induction of COX activity (measured in the presence of exogenous arachidonic acid; 30 μM) in A549 cells by different cytokines. Cells were treated with either vehicle (Control) IFNγ, IL-1β, TNFα or a mixture of IFNγ, IL-1β and TNFα (Cytmix, 10 ng ml^{-1} for all) for 12 h. The culture medium was then replaced with fresh medium containing arachidonic acid (30 μM) and incubated for 15 min. The formation of PGE_2 by the cells was then measured as an index of COX-2 activity. The data represent the mean ± s.e.mean for 3 determinations. Significant differences in the release of PGE_2 by cells treated with cytokines or vehicle were calculated by an unpaired two-tailed t test (***$P<0.001$). For abbreviations, see text.

1991) by pro-inflammatory cytokines *in vitro* and in inflamed sites *in vivo* (Vane *et al.*, 1994) supports the hypothesis (Vane, 1994) that it is this isoform of COX which is responsible for the production of prostaglandins under inflammatory conditions. The potential therapeutic benefits of inhibiting COX activity in the airways is complicated by the possibility that there is a resultant shunt of arachidonic acid to the lipoxygenase pathway (Israel *et al.*, 1993), which may be exaggerated in inflammatory conditions where phospholipase A_2 is induced (Glaser *et al.*, 1993).

There may be both deleterious and/or beneficial consequences of COX-2 induction in pulmonary epithelial cells. For instance PGE_2 increases capsaicin-induced cough in human subjects (Choudry *et al.*, 1989). However, PGE_2 dilates airway smooth muscle, inhibits the release of acetylcholine from canine bronchi (Deckers *et al.*, 1989) and inhibits mucus secretion (Marom *et al.*, 1981). PGE_2 also inhibits the release of pro-inflammatory cytokines, such as IL-1β (Monick *et al.*, 1987) and inhibits collagen synthesis by fibroblasts (Rennard *et al.*, 1986). It is, therefore, tempting to speculate that the expression of COX-2 and the subsequent release of PGE_2 may represent a natural defence mechanism of the airways to inflammation and the consequential hyperreactivity which occurs. In addition, the lipoxygenase pathway does not appear to be induced by pro-inflammatory cytokines (Masferrer *et al.*, 1990). Thus the induction of COX-2 in airway epithelial cells may result in a shunt of arachidonic acid away from the potent constrictor leukotrienes towards the production of dilator prostaglandins. However, the pro-inflammatory effects of prostaglandins released due to the induction of COX-2 in other airway cells may ultimately outweigh any beneficial effects in epithelial cells.

Both IL-1β and TNFα induced COX-2 activity, measured in the presence of excess exogenous arachidonic acid but

IFNγ alone had no effect. Interestingly, in the absence of exogenous arachidonic acid, IL-1β, but not TNFα, caused the release of COX-2 metabolites. This finding suggests that the induction of COX-2 without a concomitant induction of phospholipase A_2, results in a depletion of endogenous arachidonic acid. An interpretation of our results is that IL-1β induces both COX-2 and phospholipase A_2 whereas TNFα causes the induction of COX-2 alone. In HTBE cells, LPS plus INFγ caused a similar induction of COX-2 protein as did the cytokine mix, although detectable metabolites were released only with the latter treatment. Similarly this suggests that LPS plus INFγ induces COX-2 without sufficient induction of phospholipase A_2 whereas the cytokine mix causes the induction of both enzymes. Whether such cytokines cause the induction of COX-2 directly or via the release of other cytokines remains the subject of investigation. Interestingly cytokines such as IL-1β and TNFα are released from activated airway macrophages and may act upon the epithelium to cause the induction of COX-2 (see Barnes, 1994). Alternatively epithelial cells may generate such cytokines which then act in an autocrine manner, resulting in the induction of COX-2 (Devalia & Davies, 1993).

The therapeutic benefits of steroids in inflammatory airway disease such as asthma are well described (Barnes & Pedersen, 1993). We found that both the induction of COX-2 protein and activity were greatly reduced by dexamethasone. In addition to the dramatic suppressive effects of dexamethasone on COX-2 induction reported here, dexamethasone also limits the induction of other enyzmes which produce inflammatory mediators. Dexamethasone inhibits the induction of nitric oxide synthase in endothelial cells (Radomski *et al.*, 1990), macrophages and vascular smooth muscle (Rees *et al.*, 1990; Mitchell *et al.*, 1992). In addition dexamethasone inhibits the induction of nitric oxide synthase in human airway epithelial cells (Robbins *et al.*, 1994). Dexamethasone also inhibits the cytokine regulated induction of phospholipase A_2 (Lin *et al.*, 1992). Thus, the therapeutic benefits of dexamethasone resulting from prostaglandin suppression may be partly explained by its ability to inhibit the induction of COX-2 (this paper) and the coupled induction of phospholipase A_2 (Lin *et al.*, 1992).

Interestingly, β_2-adrenoceptor agonists, another important therapy in pulmonary inflammatory diseases, suppress the release of pro-inflammatory cytokines induced by LPS from human peripheral blood monocytes (Seldon *et al.*, 1994). Thus, glucocorticoids or β_2-agonists may inhibit the induction of enzymes such as COX-2 by modulating the release of pro-inflammatory cytokines. The continued study of the mechanisms regulating the induction of COX-2 in airway cells may provide insight into the mode of action of pro-inflammatory cytokines in diseases such as asthma and cystic fibrosis. Nonsteroid anti-inflammatory drugs such as aspirin or indomethacin are potent inhibitors of COX-1, but have less inhibitory effect on COX-2 (Mitchell *et al.*, 1993a; Meade *et al.*, 1993). These drugs have no beneficial effect in asthma suggesting that COX-1 is not important for the pathology of this disease. However, the development of selective inhibitors of COX-2 may have therapeutic potential in inflammatory pulmonary disease.

References

AKARASEREENONT, P., MITCHELL, J.A., THIEMERMANN, C. & VANE, J.R. (1994). Lipopolysaccharide induces bovine aortic endothelial cells to synthesize COX, but not nitric oxide synthase: a comparison with macrophages. *Br. J. Pharmacol.*, **111**, 33P.

BAKHLE, Y.S., MONCADA, S., DENUCCI, G. & SALMON, J.A. (1985). Differential release of eicosanoids by bradykinin, arachidonic acid and calcium ionophore A23187 in guinea-pig isolated lung. *Br. J. Pharmacol.*, **86**, 55–62.

BARNES, P.J. (1994). Airway epithelial receptors. *Eur. Resp. J.* (in press).

BARNES, P.S. & PEDERSEN, A. (1993). Ethics and Safety of inhibitor corticosteroids in asthma. *Am. Rev. Respir. Dis.*, **148**, 21–26.

CHOUDRY, N.B., FULLER, R.W. & PRIDE, N.B. (1989). Sensitivity of the human cough reflex: effect of inflammatory mediators prostaglandin E_2, bradykinin and histamine. *Am. Rev. Resp. Dis.*, **140**, 137–141.

CHURCHILL, L., CHILTON, F.H., RESAU, J.H., BASCOM, R., HUBBARD, W.C. & PROUD, D. (1989). Cyclooxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. *Am. Rev. Respir. Dis.*, **140**, 449–459.

CROXTALL, J.D. & FLOWER, R.J. (1992). Lipocortin-1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3571–3575.

CROXTALL, J.D., WAHEED, S., CHOUDHURY, Q., ANAND, R. & FLOWER, R.J. (1993). N-Terminal peptide fragments of lipocortin-1 inhibit A549 cell growth and block EGF-induced stimulation of proliferation. *Int. J. Cancer*, **54**, 153–158.

DAWSON, W., BOOT, J.R., COCKERIL, A.F., MALLEN, D.N.B. & OSBORNE, D.J. (1976). Release of novel prostaglandins and thromboxanes after immunological challenge of guinea-pig lung. *Nature*, **262**, 699–702.

DECKERS, I.A., RAMPART, M., BULT, H. & HERMAN, A.G. (1989). Evidence for the involvement of prostaglandins in modulation of acetylcholine release from canine bronchial tissue. *Eur. J. Pharmacol.*, **167**, 415–418.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

DEVALIA, J.L. & DAVIES, R.J. (1993). Airway epithelial cells and mediators of inflammation. *Resp. Medicine*, **87**, 405–407.

GLASER, K.B., MOBILIO, D., CHANNG, J.Y. & SENKO, N. (1993). Phospholipase A_2 enzymes: regulation and inhibition. *Trends Pharmacol. Sci.*, **14**, 92–98.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1974). Novel transformation of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3400–3404.

HEMPEL, S., MONICK, M.M. & HUNNINGHAKE, G.W. (1994). Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.*, **93**, 391–396.

HLA, T. & NEILSON, K. (1992). Human cyclo-oxygenase-2 cDNA. *Proc. Natl. Acad. Sci., U.S.A.*, **89**, 7384–7388.

ISRAEL, E., FISCHER, A.R., ROSENBERG, M.A., LILLY, C.M., CALLERY, J.C., SHAPIRO, J, COHN, J., RUBIN, P. & DRAZEN, J.M. (1993). The pivotal role of 5-lipoxygenase products in the reaction of aspirin-sensitive asthmatics to aspirin. *Am. Rev. Respir. Dis.*, **148**, 1447–1451.

KUJUBU, D.A., FLETCHER, B.S., VARNUM, B.C., LIM, R.W. & HERSCHMAN, H.R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclo-oxyenase homologue. *J. Biol. Chem.*, **266**, 12866–12872.

KWON, O.J., COLLINS, P.D., BARANIUK, J.N., ADCOCK, I.M. & BARNES, P.J. (1994a). Inhibition of interleukin-8 expression by dexamethasone in human cultured epithelial cells. *Immunology*, **81**, 389–394.

KWON, O.J., ROBBINS, R.A., AU, B.T., COLLINS, P.D., MAK, J.K., CHUNG, K.F. & BARNES, P.J. (1994b). Glucocorticoid inhibition of TNFα-induced interleukin-8 expression in primary cultured human airway epithelial cells. *Am. J. Physiol.*, (in press).

LEE, S.H., SOYOOLA, E., CHANMUGAM, P., HART, S., SUN, W., ZHONG, H., LIOU, S., SIMMONS, D. & HWANG, D. (1992). Selective expression of mitogen-inducible cyclo-oxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.*, **267**, 25934–25938.

LIN, L.-L., LIN, A.Y. & DE WITT, D.L. (1992). Inhibition of phospholipase A_2 expression by dexamethasone. *J. Biol. Chem.*, **267**, 23451–23454.

MAIER, J.A., HLA, T. & MACIAG, T. (1990). Cyclo-oxygenase is an immediate early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.*, **265**, 10805–10808.

MAROM, Z., SHELHAMER, J.H & KALINER, M. (1981). Effects of arachidonic acid, monohydroxyeicosatertraenoic acid and prostaglandins on the release of mucous glycoproteins from human airways *in vitro*. *J. Clin. Invest.*, **67**, 1695–1702.

MASFERRER, J.L., ZWEIFEL, B.S., SEIBERT, K. & NEEDLEMAN, P. (1990). Selective regulation of cellular cyclooxygenase by dexamethasone and LPS. *J. Clin. Invest.*, **86**, 1375–1379.

MEADE, E.A., SMITH, W.L. & DE WITT, D.L. (1993). Differential inhibition of prostaglandin endoperoxide synthase (cyclo-oxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.*, **268**, 6610–6614.

MITCHELL, J.A., AKARASEREENONT, P., THIEMERMANN, T., FLOWER, R.J. & VANE, J.R. (1993a). Selectivity of nonsteroid anti inflammatory drugs as inhibitors of constitutive and inducible cyclo-oxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 11693–11697.

MITCHELL, J.A., BELVISI, M.G., AKARASEREENONT, P., ROBBINS, R.A., KWON, O.-J., BARNES, P.J. & VANE, J.R. (1994). Induction of cyclo-oxygenase-2 in human pulmonary epithelial cells. *Br. J. Pharmacol.*, **112**, 78P.

MITCHELL, J.A., KOHLHASS, K.L., MATSUMOTO, T., POLLOCK, J.S., FORSTERMANN, U., WARNER, T.D., SCHMIDT, H.H.H.W. & MURAD, F. (1992). Induction of NADPH-dependent diaphorase and nitric oxide synthase activity in aortic smooth muscle and cultured macrophages. *Mol. Pharmacol.*, **104**, 189–219.

MITCHELL, J.A., SWIERKOSZ, T.A., WARNER, T.D., GROSS, S.S., THIEMERMANN, C. & VANE, J.R. (1993b). Regulation of prostacyclin synthesis by endogenous nitric oxide in response to bacterial lipopolysaccharide. *Br. J. Pharmacol.*, **109**, 4P.

MONICK, M., GLAZIER, J. & HUNNINGHAKE, G.W. (1987). Human alveolar macrophages suppress interleukin-1 (IL-1) activity via the secretion of prostaglandin E_2. *Am. Rev. Respir. Dis.*, **135**, 72–77.

NEIL, G.P. & FORD-HUTCHINSON, A.W. (1993). Expression of mRNA for cyclo-oxygenase-1 and cyclo-oxygenase-2 in human tissue. *FEBS*, **330**, 156–160.

O'BANION, M.K., WINN, V.D. & YOUNG, D.A. (1992). cDNA cloning and functional activity of glucocorticoid-regulated inflammatory cyclo-oxygenase. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4888–4892.

PIPER, P.J. & VANE, J.R. (1969). The release of prostaglandins from lung and other tissues. *Ann. N.Y. Acad. Sci.*, **180**, 363–385.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucorticoids inhibit the expression of inducible but not constitutive nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

REES, D.D., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone prevents the induction of nitric oxide synthase and the associated effects on vascular tone: An insight into endotoxic shock. *Biochem. Biophys. Res. Commun.*, **173**, 541–547.

RENNARD, S.I., MOSS, J., OBERPRILLER, J., HORN, B., STIER, L., OZAKI, T. & CRYSTAL, R.G. (1986). Fibroblasts: important producers and targets of inflammatory prostaglandins in the lungs. *Chest*, **83**, 92S–93S.

ROBBINS, R.A., SPRINGALL, D.R., WARREN, J.B., KWON, O.J., BUTTERY, L.K.D., WILSON, A.J., ADCOCK, I.M., RIVEROS-MORENO, V., MONCADA, S., POLAK, J. & BARNES, P.J. (1994). Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. *Biochem. Biophys. Res. Commun.*, **198**, 835–843.

SALMON, J.A. (1978). A radioimmunoassay for 6-keto-prostaglandins $F_{1\alpha}$. *Prostaglandins*, **5**, 383–397.

SELDON, P.M., BARNES, P.J. & GIEMBYCZ, M.A. (1994). Phosphodiesterase IV inhibitors and β-adrenoceptor agonists suppress lipopolysaccharide-induced tumour necrosis factor-α generation by human peripheral blood monocytes. *Br. J. Pharmacol.*, **112**, 215P.

THIEMERMANN, C., MITCHELL, J.A. & FERNS, G.A.A. (1993). Eicosanoids and atherosclerosis. *Curr. Opin. Lipidol.*, **4**, 401–406.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature*, **231**, 232–235.

VANE, J.R. (1994). Pharmacology: towards a better aspirin. *Nature*, **367**, 215.

VANE, J.R., MITCHELL, J.A., APPLETON, I., TOMLINSON, A., BISHHOP-BAILY, D., CROXTALL, J. & WILLOUGHBY, D. (1994). Inducible isoforms of cyclo-oxygenase and nitric oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 2046–2050.

XIE, W.L., CHIPMAN, J.G., ROBERTSON, D.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2692–2696.

(Received May 11, 1994
Revised June 28, 1994
Accepted July 6, 1994)